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Original Article

Determination of the sensitive stages for gonadal sex-reversal in *Xenopus laevis* tadpoles

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ABSTRACT The response of developing gonads of the clawed toad *Xenopus laevis* tadpoles to estradiol benzoate (EB) was studied between stages 44 and 67 using high resolution techniques. In presumptive genetic males the following results were obtained: 1) 100% sex reversal was induced when EB was administered before translocation of primordial germ cells (PGCs) from the gonadal epithelium into the medullary region (stages 44-50). 2) Ambiguous gonads were formed when EB treatment was initiated at stages 51-54, when PGCs were migrating into the medullary region. 3) Finally, normal testes differentiated when EB treatment began after the primordial germ cells had completed their translocation into the medulla (stages 55-56). These results suggest that EB might induce sex-reversal in genetic males by disruption of early somatic-germ cell interactions in the medullary region of the gonad. Consequently, later morphogenetic events might be deranged, preventing differentiation of testis. We propose a hypothesis in which precocious production of estradiol (E2) by genotypic females is the mechanism for primary sex differentiation.

KEY WORDS: *Xenopus laevis, gonads, sex-reversal, estradiol*

Introduction

The gonadal sex of gonochoristic species is established during early embryonic development, but the mechanisms by which this occurs are not completely understood. Lillie (1917) in his study of the freemartin effect in cattle, suggested that steroid hormones are involved in gonadal sex differentiation. Since then many studies have attempted to understand the role of steroid hormones in gonadogenesis (D'Ancona, 1951; Burns,1961; Reinboth, 1983). Administration of exogenous steroid hormones to modify the sexual differentiation of vertebrate gonads has been successful in several amphibian species. However, the results obtained from experiments with different orders and families have been irregular and sometimes contradictory (Gallien, 1950; Padoa, 1950; Witschi *et al.*, 1958).

Witschi (1950, 1967) has interpreted the results of sex-reversal induced by exogenous steroids in terms of his hypothesis of a "corticomedullary antagonism" which involves two non-steroidal inducers: "medullarin" and "corticin". He postulated that exogenous steroids have an indirect teratogenic effect and that endogenous steroid hormones do not play a natural role in gonadal differentiation. On the other hand, it was recently shown that exogenous estradiol can induce ovarian differentiation in reptile species in which temperature affects sex differentiation (Pieau, 1974; Gutzke and Bull, 1986; Bull *et al.*, 1988; Dournon *et al.*, 1990). It was proposed that in these species endogenous steroid hormones may play a natural role in gonadal sex differentiation (Raynaud and Pieau, 1985; Crews *et al.*, 1989).

The present study addresses these two hypotheses by investigating the effect of estradiol benzoate on gonadal morphogenesis in the clawed toad, *X. laevis*, a species exhibiting complete and functional sex-reversal of genetic males (Gallien, 1953).

Results

The genetic sex of *X. laevis* belongs to the system ZZ/ZW. Gonadal sex differentiation is reached at stage 56. In genetic males the cortex becomes a thin epithelium devoid of primordial germ cells (PGCs) and is separated from the medulla by connective tissue (Fig. 1A). In contrast, gonads of genetic females retain the PGCs among the epithelial cells of the cortex, and the medullary cells form a flat epithelium surrounding a central cavity (Fig. 1B). The results of EB-treated tadpoles are given in Table 1. When treatment was initiated at stages 44-50 (Groups 1-4) all survivors had ovaries after three months, suggesting that all gonads of presumptive genetic males were induced to undergo sex-reversal. Morphologically it was found

Abbreviations used in this paper: EB, estradiol benzoate; PGCs, primordial germ cells; E2, estradiol.

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that no genital ridges were present between stages 44-48. At stages 49-50, the genital ridges developed as two evaginations of the coelomic epithelium where the first PGCs have arrived (Fig. 1C). In samples from Groups 1-4 fixed after one month of EB treatment no morphological abnormalities were observed as compared with controls.

At stages 51-53 an undifferentiated gonad was established. Some cells of the coelomic epithelium migrated towards the interior of the genital ridge forming the gonadal medulla, and the PGCs remained in the surface epithelium (Figs. 1D). Furthermore, in presumptive genetic males, PGCs migrated from the cortex into the medulla at stages 54-55. When treatment was initiated between stages 51-54, around 50% of survivors had normal ovaries; however, the other 50% had abnormal gonads qualified as "ovotestes" (Table 1, Groups 5-8). They were characterized for the presence of PGCs in both the cortex and the medulla (Figs. 1E and F). Since in control groups presumptive genetic males had all PGCs in the medulla (Fig. 1A), it was assumed that the "ovotestes" correspond to gonads of presumptive genetic males in which normal development was disturbed.

Finally, when EB treatment began at stages 55-56, in which all PGCs appeared in the medullary region of presumptive genetic males, the ovaries-testes ratio was close to 1:1 (Table 1, Groups 9 and 10), as in control tadpoles. These results suggested that at these stages, the steroid had not affected normal differentiation of testes in presumptive genetic males. Control tadpoles in which only ethanol was added had a survival rate of approximately 50% after three months, quite similar to tadpoles under EB treatment (Table 1). The gonads of controls differentiated either as ovaries or as testes in a 1:1 ratio and no ovotestes were found.

Discussion

Witschi (1950, 1967) and Chang and Witschi (1955) evaluated the action of exogenous sex steroids on developing gonads of different species of amphibians and interpreted the results in terms of a "corticomedullary antagonism". They postulated that these hormones acted as teratogens, either destroying or inhibiting one of the two gonadal territories: the medulla or the cortex. Therefore, destruction of the medulla by EB in presumptive genetic males of *X. laevis* induced sex reversion by allowing the cortex to form an ovary. Since in the present study no evidence of cell death or of any other structural alterations was found in medullary cells under EB treatment, Witschi's notion of selective destruction of the medullary cells is not supported.

However, our finding of complete male sex-reversal by EB before or during the early establishment of the gonadal medulla (stages 44-50) may be explained according to Witschi's idea of an inhibition of medullary differentiation as follows: when EB is added, before translocation of epithelial and primordial germ cells (PGCs) into the medullary region, the presence of this hormone might prevent the differentiation of male PGCs and somatic medullary cells. Therefore, migration of PGCs from the coelomic epithelium into the medulla might also be prevented. At stages 51-54, when presumptive medullary cells have migrated into the central region of the gonad and PGCs in turn are undergoing translocation, EB appears to interrupt this process and different degrees of ovotestes are formed. Finally, from stage 55 onwards, when PGCs have finished their translocation into the medulla and this region becomes completely segregated from the cortex, EB treatment is unable to induce male sex-reversal.

Since in our present observations EB appears to inhibit translocation of PGCs, no interaction of these cells with somatic medullary cells can be responsible for preventing testes differentiation. Inhibition by EB of somatic-germ cell interactions in the medullary region of genetic males might be due to two different primary effects: a) somatic medullary cells are inhibited from producing a chemotactic substance responsible for translocation of PGCs. b) Sex differentiation of PGCs is prevented, making them unable to migrate into the medullary region.

For primary effect a) there is no evidence that medullary cells of genetic males produce a chemotactic substance. However, in the present and previous studies (Merchant-Larios and Villalpando, 1981) it has been observed that medullary establishment in undifferentiated gonads of *X. laevis* depends upon migration of some epithelial cells from the coelomic epithelium. In presumptive genetic males, there is further migration of PGCs from this region into the medulla. Therefore, it is reasonable to postulate the existence of such a chemotactic substance.

As regards primary effect b), it has been demonstrated that in *X. laevis* the patterns of proliferation and differentiation of PGCs differ in males and females (Kalt, 1973; Ijiri and Egami, 1975) and that they precede histological sex differentiation of the gonad (Zust and Dixon, 1977). Thus, inhibition of these processes by EB might prevent testis differentiation.

Although the present results help to understand the histological conditions of gonads in which EB induces male sex-reversal in *X. laevis*, the role of steroid hormones during normal sex differentiation is not clear. The failure of testosterone to induce sex-reversal in genotypic females of *X. laevis* (Witschi, 1950; Gallien, 1956, 1959) and the finding that this effect is induced by testicular grafts (Mikamo and Witschi, 1963) have led Witschi to discount the role of sex steroids in gonadal sex differentiation in normal developing tadpoles. On the basis of our present results a hypothesis can be

Fig. 1. Semi-thin sections (1 μm) of *X. laevis* tadpole gonads at various developmental stages. (A) *Male gonad from an untreated tadpole at stage 56. Primordial germ cells (pgc) are present in the medullary region (m). The cortex (c) is formed by a thin epithelium devoid of primordial germ cells. Bar= 28 μm.* **(B)** *Female gonad at stage 56 taken from a control tadpole. The medullary cells (m) have formed a wide cavity and the primordial germ cells (pgc) are present only in the cortex (c). Bar= 28 μm.* **(C)** *At stage 49 the first primordial germ cells (pgc) form an evagination of the coelomic epithelium at each side of the hindgut mesentery (hm). Parts of the mesonephros (ms) and dorsal aorta (da) are shown. Bar= 13 μm.* **(D)** *At stage 53 typical undifferentiated gonads are established. Although in this section the medulla (m) appears only in one gonad, serial sections demonstrated medullary tissue in both gonads. The dorsal aorta (da) containing numerous erythroblasts and part of the mesonephros (ms) are shown. Bar= 15 μm.* **(E)** *and* **(F)** *These figures show two different ambiguous gonads considered as "ovotestes" at stage 56. They correspond to tadpoles treated with EB at stages 52 and 54 respectively. Primordial germ cells (pgc) are abnormally distributed in both the cortex (c) and the medulla (m).* **(E)** *has a compact testis-like medullary region and a primordial germ cell in the cortex (c) undergoing mitosis.* **(F)** *shows a gonad with cavitated ovarian-like medullary region. However several primordial germ cells (pgc) are associated to the epithelial cells of the medulla. This abnormal position of primordial germ cells was never observed in gonads of control tadpoles. E: Bar= 7 μm; F: Bar= 30 μm.*

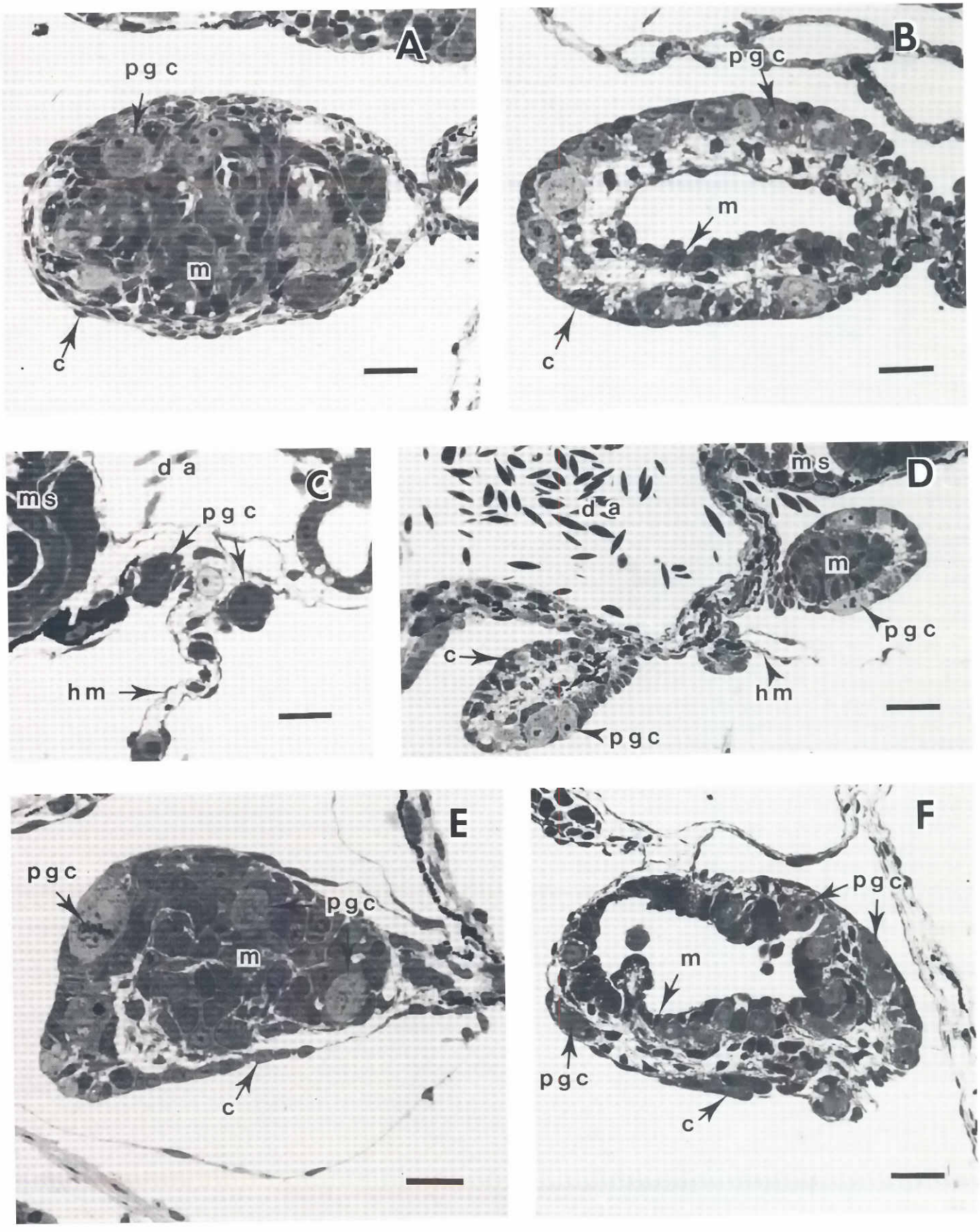
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TABLE 1

EFFECTS OF ESTRADIOL BENZOATE ON GONADAL DEVELOPMENT OF *XENOPUS LAEVIS* TADPOLES

	Initiation of Treatment		Gonadal Sex at Stage 56-67			
Group	Developmental Stage	Gonadal Stage	Ovaries	Testes	Ovotestes	Survivors*
1	44	-	66	0	0	66
2	48	-	68	0	0	68
3	49	Genital ridge	55	0	0	55
4	50	Genital ridge	60	0	0	60
5	51	Early medulla	25	0	28	53
6	52	Early medulla	27	0	22	49
7	53	Early medulla	29	0	31	60
8	54	Cortex and medulla	30	0	35	65
9	55	Cortex and medulla	28	24	0	52
10	56	Sex differentiation	23	26	0	49

Note. (*) Numbers of survivors are equivalent to percentage since each treated group started with 100 tadpoles.

proposed: there is an asynchronous production of estradiol (E2) in genotypic female and male tadpoles. The former begin to produce E2 before stage 50, inhibiting the differentiation of the medullary cells. Thus, PGCs remain in the cortex and an ovary is formed. In contrast, genotypic males do not produce E2 before stage 50 and male PGCs and medullary cells differentiate "constitutively" and a testis is formed. Furthermore, in genotypic males medullary cells produce a diffusible non-steroidal factor that inhibits the cortex and is able to induce female sex reversion when grafted.

To support this hypothesis, precocious production of E2 in genetic females has to be demonstrated. Lack of heteromorphic sex chromosomes in *X. laevis* make this demonstration a difficult task. Nevertheless, a comparison of steroidogenesis in all male tadpoles (offspring of sex-reversed males and normal males) with a normal tadpole population (1:1 sex ratio) may answer this question. Failure to produce E2 before stage 50 by genotypic females, or simultaneous production of E2 by genotypic male and female tadpoles, would refute this hypothesis.

Complete sex-reversal of presumptive genetic males by EB before stage 50 and the partial effect of this hormone at stages 51 to 54 provided some support for the "dual gonadal inducer" hypothesis of Witschi. However, in our present hypothesis, E2 represents "corticin", although the chemical nature of "medullarin" still remains unknown.

Materials and Methods

Sexually mature male and female *X. laevis* were stimulated with human chorionic gonadotropin to induce gamete liberation (New, 1966). Ten groups of 100 tadpoles each were reared in plastic boxes with 7 l of tap water which was changed three times a week. Treatment with estradiol benzoate (EB) dissolved in ethanol at a final concentration of 100 µg/l of water was initiated at different developmental stages (Normal Table of Nieuwkoop and Faber, 1956): in group one, EB treatment was initiated at stage 44. In groups 2-10, EB treatment began at stages 48-56 respectively (Table 1). Two control groups of 100 tadpoles each were kept under the same conditions as the experimental groups, except that only ethanol was added (100 µl/l of water). The photoperiod was kept at 12 h light and 12 h darkness. The tadpoles were fed with food from Caroline Biological Supply (U.S.A) or powdered alfalfa leaf.

After around three months of EB treatment (stages 56-67, depending upon initiation of treatment) the surviving tadpoles were sacrificed and their gonadal sex was histologically determined. To establish the state of the gonads before and during EB treatment, 10 samples from each group were fixed at the beginning and after one month. Gonads attached to mesonephros were fixed with Kalt and Tandler's (1971) solution omitting the acrolein. They were postfixed in 1% OsO4 in 0.1 M cacodylate buffer, dehydrated in acetone and embedded in Epon 812. Semi-thick sections of (1 µm) were stained with 0.5 % toluidine blue.

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